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Analysis of the subunit assembly of the type IC restriction–modification enzyme *EcoR124I*

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ABSTRACT

Type I restriction–modification (R–M) enzymes are composed of three different subunits, of which HsdS determines DNA specificity, HsdM is responsible for DNA methylation and HsdR is required for restriction. The HsdM and HsdS subunits can also form an independent DNA methyltransferase with a subunit stoichiometry of M_2S_1 . We found that the purified *EcoR124I* R–M enzyme was a mixture of two species as detected by the presence of two differently migrating specific DNA–protein complexes in a gel retardation assay. An analysis of protein subunits isolated from the complexes indicated that the larger species had a stoichiometry of $R_2M_2S_1$ and the smaller species had a stoichiometry of $R_1M_2S_1$. *In vitro* analysis of subunit assembly revealed that while binding of the first HsdR subunit to the M_2S_1 complex was very tight, the second HsdR subunit was bound weakly and it dissociated from the $R_1M_2S_1$ complex with an apparent K_d of $\sim 2.4 \times 10^{-7}$ M. Functional assays have shown that only the $R_2M_2S_1$ complex is capable of DNA cleavage, however, the $R_1M_2S_1$ complex retains ATPase activity. The relevance of this situation is discussed in terms of the regulation of restriction activity *in vivo* upon conjugative transfer of a plasmid-born R–M system into an unmodified host cell.

INTRODUCTION

Type I restriction and modification (R–M) systems protect the bacterial cell against invasion of foreign DNA (such as viruses) by cleaving DNA which lacks a target specific N6-adenine methylation. The second physiological role of these systems is to restore full methylation of the target sites on the host DNA after DNA replication. Type I R–M enzymes are composed of three different subunits (HsdR, HsdM and HsdS) encoded by the three *hsd* genes (for recent reviews see 1,2). All three subunits are absolutely required for restriction activity, while the HsdM and HsdS subunits are sufficient for modification activity and can also form an independent DNA methyltransferase (MTase). Type I R–M enzymes specifically recognise a non-palindromic DNA sequence (e.g. GAAnnnnnnRTCG for *EcoR124I*, where n is any base and R is a purine) but cleave DNA at a non-specific site distant from the

recognition sequence using Mg^{2+} , S-adenosylmethionine (AdoMet) and ATP as cofactors (3,4). Binding of the endonuclease to a non-modified recognition site activates a powerful ATPase activity (5), which fuels DNA translocation past the DNA–enzyme complex, while the enzyme remains bound to the recognition site (6,7). DNA is cleaved at positions where the DNA translocation stops either due to a collision of two translocating enzyme molecules on two-site, linear DNA substrates (8,9), or due to the build-up of topological strain on circular molecules (10). The endonuclease does not turnover in the cleavage reaction, however, the ATPase activity continues for a long period of time after the cleavage is completed (3,5). DNA methylation activity of the type I R–M systems results in a transfer of a methyl group from AdoMet to the N-6 position of a specific adenine in each strand of the recognition sequence (11,12).

Type I R–M systems are grouped into four families based on allelic complementation, protein homologies and biochemical properties of the enzymes. Types IA, IB and ID R–M systems are chromosomally encoded (13,14) while most type IC R–M systems are carried on large conjugative plasmids (15–17). The type IA family is typified by the *EcoKI* and *EcoBI* enzymes, type IB by *EcoAI* and type IC by *EcoR124I*. *EcoKI* forms a stable $R_2M_2S_1$ complex; the independent *EcoKI* MTase (M_2S_1) is, however, a relatively weak complex, dissociating into an inactive M_1S_1 species and free HsdM subunit (18–20). The purified *EcoBI* restriction endonuclease has been described as existing in a number of different stoichiometric forms including $R_2M_2S_1$, $R_1M_2S_1$ and $R_1M_1S_1$ (21). The type IB restriction endonuclease *EcoAI* was found to be a weak complex that dissociated into MTase and HsdR subunit when purified, and the active endonuclease could only be studied following assembly *in vitro* (22). We have recently purified the *EcoR124I* restriction endonuclease from a cell culture harbouring a recombinant, two-plasmid system, which over-produces all three subunits. The stoichiometry of this endonuclease preparation appeared to be $R_1M_2S_1$ (23). The *EcoR124I* MTase has also been over-produced and purified to homogeneity (12,24). This enzyme was found to exist only in the M_2S_1 stoichiometry and no M_1S_1 complex has been detected (12).

The *hsd* genes of all R–M systems can be transferred into an unmodified host by conjugation, transformation or transfection despite the fact that the presence of a restriction enzyme in the recipient cell would be a lethal event (17,23,25–31). It has been reported that the appearance of restriction activity was delayed

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after conjugative transfer of *EcoKI* genes into an unmodified host, while the modification activity was expressed immediately after the conjugation (29). It was proposed that restriction and modification activities of the *EcoKI* R–M system are regulated by a gene distant from the *hsd* locus since the conjugative transfer of *EcoKI hsd* genes into a mutant form of *Escherichia coli* C (designated as *hsdC*) had a lethal effect on the cell (29). The same regulation mechanism was found to be employed in the type IB R–M systems (32). However, the conjugal transfer of *EcoR124I* (IC) *hsd* genes into the *hsdC* mutant was not lethal and full restriction activity was detected six generations after the start of conjugation (32). Recently, it has been shown that ClpX and ClpP, components of the ClpXP protease, are necessary for efficient transmission of genes encoding the *EcoKI* and *EcoAI* R–M systems, suggesting protease-mediated post-translational control of restriction and modification (33). The involvement of protease control of restriction and modification would support the proposal that post-translational control of restriction activity of *EcoKI* could be related to subunit assembly (20).

In this paper we show that the purified *EcoR124I* restriction endonuclease is a mixture of two species, which have a subunit stoichiometry of $R_2M_2S_1$ and $R_1M_2S_1$, respectively. Only the former species was found to have endonuclease activity. However, the $R_2M_2S_1$ complex is relatively weak, dissociating into free HsdR subunit and the restriction-deficient $R_1M_2S_1$ assembly intermediate, which appears to be a very tight complex. We discuss the relevance of this situation with respect to the propagation of a conjugative plasmid-borne R–M system into an unmodified host cell. We suggest that control of *EcoR124I* endonuclease activity *in vivo* is at the level of subunit assembly in a manner similar to that observed for *EcoKI* (20).

MATERIALS AND METHODS

DNA substrates

Complementary HPLC-purified oligonucleotides used in the gel retardation assays were purchased from Cruachem Ltd. The *EcoR124I* recognition site is shown in bold (top strand: 5'-CTACGGTACCGAAACGCGT**GTCTGGGCCCCGCGAAGC**-TTGC-3'), DNA concentration was determined from the absorption at 260 nm. The extinction coefficients of the oligonucleotides and the annealed oligoduplex were calculated as a sum of the contributions from individual nucleotides, taking into account hyperchromicity observed after digestion of the DNA to completion with snake venom phosphodiesterase (34). The oligoduplex (usually 2.5 µg) was 5'-end-labelled using [γ - 32 P]ATP (25 µCi) and T4 polynucleotide kinase. Unincorporated label was removed using 'NucTrap' columns (Stratagene).

A 2891 bp plasmid containing a single *EcoR124I* recognition site, pDRM-1R, (23) used in DNA cleavage assays was produced from *E. coli* HB101 (35) and its covalently closed form was purified by an equilibrium centrifugation in a CsCl–ethidium bromide gradient (36).

Protein preparations

The *EcoR124I* restriction endonuclease, MTase (M_2S_1) and HsdR subunit were over-produced and purified as described previously (12,23). Either the plasmid pACR124 or pBGSR124 containing the *hsdR* gene under the control of the P_{trc} promoter (23), were used together with the MTase plasmid pJS4M (24) to

over-produce the endonuclease. The latter plasmid combination produced a higher concentration of HsdR than the former, presumably due to the higher copy number of pBGSR124. Both two-plasmid systems produced the same concentrations of the HsdS and HsdM subunits (data not shown). To obtain a stable clone containing the pJS4M-pBGSR124 plasmid-expression system, the cells were first transformed with the pJS4M plasmid (MTase) and then with pBGSR124 plasmid (HsdR). Transformation of pJS4M and pBGSR124 at the same time was found to be lethal. The molar concentration of purified proteins was determined from the absorbance at 280 nm using molar extinction coefficients calculated as a sum of contributions from tyrosines and tryptophans in the predicted amino acid sequences [HsdR subunit; 91 900/M/cm, MTase (M_2S_1); 160 400/M/cm, endonuclease, 252 300/M/cm for $R_1M_2S_1$, 344 200/M/cm for $R_2M_2S_1$].

Reconstitution of the endonuclease

Fully active reconstituted *EcoR124I* endonuclease, used in the DNA cleavage and gel retardation assays, was simply produced by mixing of the purified MTase (M_2S_1) and HsdR subunit in assay reaction buffers (see below). The concentration of active endonuclease was taken as the input concentration of the MTase.

Determination of subunit ratio in the endonuclease–DNA complexes

The two DNA–protein complexes observed with purified and reconstituted *EcoR124I* endonucleases (Fig. 1) were separated in preparative amounts (2 µM 39mer DNA in 10 µl binding reaction) on a non-denaturing polyacrylamide gel. After staining the gel in ethidium bromide solution, fluorescent bands were excised from the gel, mashed and protein extracted with SDS–PAGE loading buffer. Protein extracts were subjected to SDS–PAGE together with a series of R/MTase mixtures of known molar ratios. After staining with Coomassie Blue R250, the gel was quantified by densitometry using Image Grabber 2.05b1 and NIH image 1.52 software. The ratio of densitometer traces of individual subunits (S:M:R) was calculated for each sample. To determine the stoichiometry of the species isolated of the DNA–protein complexes, the best fit between their S:M:R ratios and one of the values obtained with the R/MTase mixtures was determined.

HPLC gel filtration

HPLC gel filtration used a Rainin Dynamax 4.6 × 250 mm Hydropore-5-SEC column and a guard column. Samples of 20 or 50 µl, in 20 mM Tris, 20 mM 2-[N-morpholine] ethane sulphonic acid (MES), 0.2 M NaCl, 10 mM MgCl₂, 7 mM β-mercaptoethanol, 0.1 mM EDTA, pH 6.5, were injected onto the column. The samples were equilibrated at room temperature for varying time periods up to 24 h prior to injection. The flow rate was 0.5 ml/min and detection was most sensitive and stable at 254 nm. The column was calibrated with a series of globular proteins of known molecular weight giving a linear calibration curve of log (molecular weight) as a function of elution time. Most of our samples after buffer exchange into the column buffer using PD10 Sephadex G50 columns (Pharmacia), contained trace amounts of a small molecule which we believe to be glycerol. This gave rise to a 'solvent peak' after ~6.5 min, the elution time of which served as an internal standard to correct for slight run to run variation in protein elution times. The apparent molecular weight of the proteins eluting from the column was calculated. In the

titration of MTase with HsdR subunit, the HsdR subunit–MTase complex was present only as a partially resolved shoulder when HsdR subunit was in large excess of the MTase.

DNA cleavage and ATPase assays

DNA cleavage and ATPase activities of *EcoR124I* restriction endonuclease were assayed using plasmid pDRM-1R (single *EcoR124I* site) as described previously (23). DNA, usually at a concentration of 200 nM, was incubated either with an equimolar concentration of the MTase and increasing concentration of the HsdR, or with a range of concentrations of reconstituted endonuclease. After addition of ATP, the reactions were incubated for 5 min (sufficient time to reach the reaction endpoint). DNA cleavage activity (concentration of full-length linear plasmid DNA) and ATPase activity (concentration of inorganic phosphate) were measured as described previously (23).

Gel retardation assay

DNA binding reactions were usually performed in a volume of 10 μ l in the presence of a buffer consisting of 50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT and 10% (v/v) glycerol. The end-labelled 39mer oligoduplex (see above) of known concentration was incubated with purified endonuclease, MTase and MTase/HsdR or endonuclease (ENase)/HsdR mixtures over a range of various concentrations and/or protein:protein ratios, respectively, at room temperature for 10 min. Unbound DNA and DNA–protein complexes were separated on a 5% non-denaturing polyacrylamide gel run, at 4°C, at 100 V in 40 mM Tris-acetate pH 8.0, 1 mM EDTA buffer (TAE). After electrophoresis, gels were dried and subjected to autoradiography or quantified using a PhosphorImager and Image Quant software.

RESULTS

Gel retardation analysis of *EcoR124I* endonuclease reveals two molecular species

We have previously purified the *EcoR124I* endonuclease following over-production of the protein by a recombinant, two-plasmid system consisting of pJS4M (MTase) and pACR124 (HsdR). Using a combination of data from gel filtration and densitometric scanning SDS–polyacrylamide gels stained with Coomassie Blue, we concluded that this enzyme had a stoichiometry of R₁M₂S₁ (23).

To investigate the DNA-binding properties of the over-produced *EcoR124I* endonuclease, the technique of gel retardation, with a 39mer oligoduplex carrying a single *EcoR124I* recognition site, was utilised. Unexpectedly, two DNA–protein complexes were observed after separation of bound and unbound DNA on a non-denaturing polyacrylamide gel (Fig. 1, lane 2). The complex with faster mobility (complex I) was preferentially formed (88% of bound DNA). When the plasmid pBGR124 (23), a higher copy number plasmid, which produces a significantly higher level of HsdR than the plasmid pACR124, was used with the plasmid pJS4M in the two-plasmid over-producing system, the enzyme preparation showed a different ratio between the two complexes; 53% of complex I and 47% of complex II (Fig. 1, lane 4). To exclude non-specific DNA binding, the experiment was repeated in the presence of excess competitor DNA (poly dI–dC), but no change was observed in the two retarded DNA bands (data not shown).

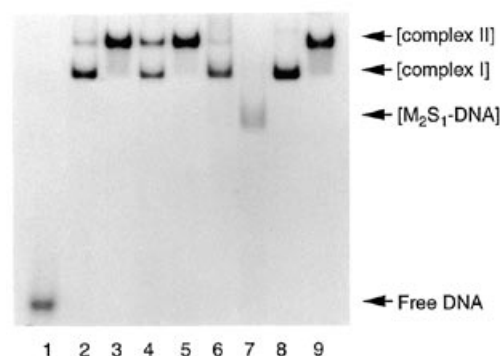


Figure 1. Gel retardation analysis of purified *EcoR124I* restriction endonuclease. DNA binding reactions were carried out with 200 nM 39mer oligoduplexes as described in Materials and Methods. Lane 1, free DNA; lane 2, 200 nM endonuclease over-produced by the pJS4M-pACR124 plasmid system; lane 3, as lane 2 plus 1 μ M HsdR; lane 4, 200 nM endonuclease over-produced by the pJS4M-pBGR124 plasmid system; lane 5, as lane 4 plus 1 μ M HsdR; lane 6, as lane 4 plus 200 nM MTase; lane 7, 200 nM MTase; lane 8, 200 nM MTase plus 200 nM HsdR; lane 9, 200 nM MTase plus 1200 nM HsdR. Positions of individual DNA–protein complexes and unbound DNA on the gel are indicated.

Another possibility was that there were two stoichiometric species present in the endonuclease preparations. This was confirmed by the following results. Addition of excess HsdR to both endonuclease preparations resulted in the disappearance of the complex I and increased the concentration of the complex II (Fig. 1, lanes 3 and 5). A 1:1 mixture of HsdR and the MTase (M₂S₁) produced only complex I, while the 6:1 mixture of HsdR and the MTase produced mostly complex II (Fig. 1, lanes 8 and 9). Finally, addition of the MTase to the mixture of the endonuclease and DNA led to the disappearance of complex II and increased the amount of complex I (Fig. 1, lane 6). These results indicate that the two observed species vary in the number of HsdR subunits, and the lower molecular weight species is preferentially formed at HsdR:MTase ratios <1.

Analysis of subunit stoichiometry in protein–DNA complexes of *EcoR124I*

To investigate the stoichiometry of the two species present in the endonuclease preparations, the proteins bound in complexes I and II were extracted from the retardation gel and subjected to an SDS–PAGE analysis together with a series of R/MTase mixtures, including ratios 0.5:1, 1:1, 2:1, 3:1 and 4:1. The relative ratio of the three endonuclease subunits, in each sample, was determined from a densitometer scan of the polyacrylamide gel after staining the gel with Coomassie Blue (23). As can be seen in Figure 2, the ratio of subunits in the protein extracted from the complex I agrees with the subunit ratio in the 1:1 mixture of HsdR and MTase and the protein from complex II corresponds with the 2:1 mixture. Thus the protein species bound in complex I has a stoichiometry of R₁M₂S₁ and that bound in complex II has a stoichiometry of R₂M₂S₁.

Analysis of interaction between the MTase and HsdR in the presence of DNA

To investigate the endonuclease assembly *in vitro*, we performed a reconstitution titration of the MTase (M₂S₁) with the HsdR subunit, and followed this by a gel retardation assay using a 39mer oligoduplex carrying the *EcoR124I* recognition site

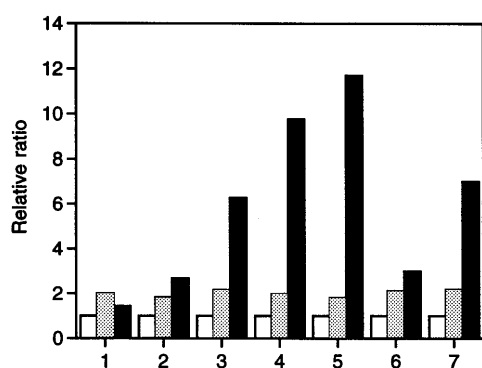


Figure 2. Subunit composition of the gel-retarded protein–DNA complexes of *EcoR124I*. The histograms compare relative ratios of subunits extracted from the two DNA–protein complexes of purified *EcoR124I* endonuclease produced by the pJS4M-pBGSRI24 plasmid system (complexes I and II shown in Fig. 1) to the relative ratios of subunits in a series of mixtures of HsdR and MTase, as determined by densitometric scanning of SDS–PAGE gels stained with Coomassie Blue. White bars, HsdS; grey bars, HsdM; black bars, HsdR. Panels 1–5 on the x-axis correspond to the following HsdR:MTase ratios: 0.5:1, 1:1, 2:1, 3:1, 4:1. Panels 6 and 7 are complex I and complex II, respectively. The data suggest that the stoichiometry of the species in complex I is $R_1M_2S_1$, and the stoichiometry of the species in complex II is $R_2M_2S_1$.

(Fig. 3). As can be seen, increased concentration of HsdR resulted in the disappearance of the M_2S_1 –DNA complex and appearance of complex I. At an HsdR:MTase ($R:M_2S_1$) molar ratio of 1, only complex I was present. This suggests that one of the two R-binding sites in the MTase is stronger than the other one since in the case of two equivalent binding sites, at an HsdR:MTase ratio of 1, 50% MTase would be occupied with one HsdR subunit, 25% would be occupied with two HsdR subunits and 25% MTase would bind no HsdR subunit. Equivalent binding sites for HsdR have been observed for *EcoKI* (L.Powell and D.Dryden, unpublished observations). The $R_2M_2S_1$ –DNA complex started to appear only when the HsdR:MTase ratio was >1 ; while under these conditions the $R_1M_2S_1$ –DNA complex disappeared. However, the $R_2M_2S_1$ –DNA complex was not fully present at an HsdR:MTase molar ratio of 2, and even above the molar ratio of 3 there was still a smear on the gel, lying between the two complexes. This suggests that the $R_2M_2S_1$ species is a much weaker complex than the $R_1M_2S_1$ species and that the smear corresponds to its dissociation into $R_1M_2S_1$ and HsdR within the gel matrix.

In order to measure an apparent dissociation constant characterising the equilibrium between $R_1M_2S_1$ and $R_2M_2S_1$ species, the subunit complexes present in a series of dilutions of a 1:2 mixture

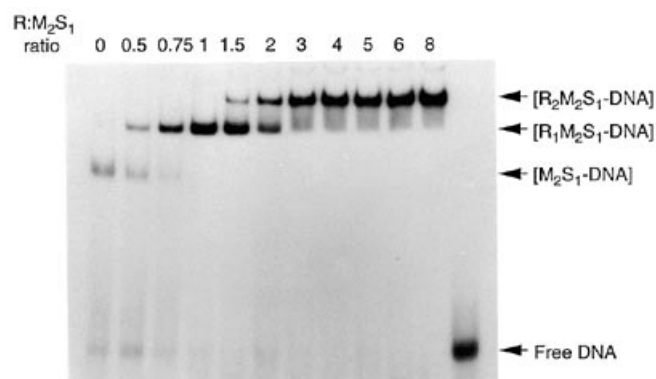


Figure 3. Titration of *EcoR124I* MTase with HsdR followed by gel retardation assay. A mixture of 200 nM MTase and 200 nM 39mer oligoduplex, containing one *EcoR124I* recognition site, was incubated with increasing concentrations of HsdR subunit at room temperature for 10 min. DNA–protein complexes and free DNA were separated on a non-denaturing polyacrylamide gel as described in Materials and Methods. The HsdR:MTase ratio in individual reactions and the position of DNA–protein complexes on the gel are indicated.

of the MTase and HsdR, starting from a protein concentration of 1 mM, were analysed by gel retardation assay in the presence of 20 nM 39mer oligoduplex. Figure 4a shows that at a protein concentration of 1 mM, the $R_2M_2S_1$ species was almost exclusively present. However, a faint band appeared on the top of the gel at high protein concentrations apparently due to protein aggregation or non-specific binding of a second endonuclease molecule. When the protein was diluted, the $R_2M_2S_1$ species dissociated to $R_1M_2S_1$ (seen as an increased concentration of the $R_1M_2S_1$ –DNA complex) which appeared to be stable in the concentration range used in the experiment (10^{-6} – 10^{-9} M). The retardation gel was quantified (Materials and Methods) and the percentage of the $R_2M_2S_1$ –DNA complex of the total bound DNA was plotted as a function of protein concentration (Fig. 4b). Only data for protein concentrations >20 nM were used for plotting since at lower concentrations dissociation of the DNA–protein complex occurred (Fig. 4a). An apparent equilibrium dissociation constant of the $R_2M_2S_1$ complex was determined as the concentration of enzyme where 50% of $R_2M_2S_1$ –DNA was dissociated to $R_1M_2S_1$ –DNA. The value was estimated at $\sim 2.4 \times 10^{-7}$ M. Similar dilution experiments were also performed with a 1:1 mixture of MTase and HsdR (containing only $R_1M_2S_1$). No dissociation of $R_1M_2S_1$ complex into the MTase and HsdR was observed even at the lowest protein concentrations detectable by the gel retardation assay (10^{-9} M) (not shown). Therefore, the $R_1M_2S_1$ species is apparently a very stable complex.

Table 1. Molecular weights of complexes found for the *EcoR124I* system

Sample	Apparent molecular weight by HPLC gel filtration (kDa)	Expected molecular weight (kDa)
M_2S_1 MTase	220–230	162.2
HsdR subunit	137–148	119.6
<i>In vivo</i> assembled $R_1M_2S_1$ ^a	280–300	281.8
<i>In vivo</i> assembled mixture of $R_1M_2S_1$ + $R_2M_2S_1$ ^b	333–350	281.8 + 401.5
<i>In vitro</i> assembled $R_2M_2S_1$ (freshly prepared)	290–315	401.5
<i>In vitro</i> assembled $R_2M_2S_1$ (after 24 h incubation)	380–420	401.5

^aProduced by the pJS4M-pACR124 plasmid system.

^bProduced by the pJS4M-pBGSRI24 plasmid system.

Analysis of interactions between the MTase and HsdR in the absence of DNA

To analyse endonuclease assembly in the absence of DNA, we used HPLC gel filtration. The apparent molecular weights of various subunit complexes of *EcoR124I* from an HPLC gel filtration column were determined from their elution times and are given in Table 1. It can be seen that the MTase and HsdR subunit eluted from the column earlier than expected, giving a slightly higher weight. Similar behaviour was observed for the *EcoKI* enzyme (20). The endonuclease purified from cells containing the pJS4M-pACR124 plasmid-expression system eluted with an apparent molecular weight similar to that expected for $R_1M_2S_1$. This is in agreement with previously published data (23). The endonuclease purified from cell culture containing the pJS4M-pBGSR124 plasmid-expression system had an apparent mass intermediate between $R_1M_2S_1$ and $R_2M_2S_1$ and formed a slightly asymmetric peak suggesting that it was a mixture of these two species. A freshly prepared mixture of MTase with a 5-fold excess of HsdR subunit, eluted with an apparent weight close to that expected for $R_1M_2S_1$. However, the same mixture after a 24 h incubation at room temperature showed the formation of a higher molecular weight species with a weight equal to that expected for the $R_2M_2S_1$ endonuclease. Figure 5a shows the elution profiles of the HsdR subunit, MTase and a mixture of these two components freshly prepared and after 24 h. The titration of a constant concentration of MTase with the HsdR subunit also showed the dramatic change in molecular weight of the complex upon prolonged incubation (Fig. 5b). Therefore, HPLC data indicated that the formation of the $R_2M_2S_1$ complex is a slow process under the conditions used. One possible explanation of this observation is that the presence of 5% glycerol in the samples slows diffusion; although it seems unlikely that this would be a large effect. Alternatively, it appears likely that the presence of DNA in the sample grossly changes the rate of attainment of thermodynamic equilibrium. This idea is supported by the fact that DNA cleavage appeared in a very short time following mixing of MTase and HsdR in the presence of DNA (see the next section).

DNA cleavage and ATPase activities of MTase-HsdR complexes

To investigate enzyme activities of the $R_1M_2S_1$ and $R_2M_2S_1$ species, DNA cleavage and ATP hydrolysis were assayed following a reconstitution titration of the MTase with HsdR in the presence of a plasmid DNA substrate. The DNA used contained a single *EcoR124I* recognition site. This DNA readily undergoes primary cleavage to produce full-length linear DNA. Secondary processing of the linear product is observed only at very high enzyme:DNA ratios (23). Reaction products (linear DNA and inorganic phosphate, respectively) were quantified by densitometric scanning of agarose gels and malachite green assay as described elsewhere (23). A gradually increasing concentration of HsdR subunit was added into a 1:1 mixture of the MTase and DNA, which were present at a concentration of 200 nM to ensure full DNA binding. The same concentration conditions were also used in the reconstitution titration followed by a gel retardation assay (Fig. 3). At an HsdR:MTase ratio <1 , where only the $R_1M_2S_1$ complex was detected by a gel retardation assay, no DNA cleavage occurred (Fig. 6a). However, ATPase activity gradually increased with HsdR concentration (Fig. 6b). It is important to note that the

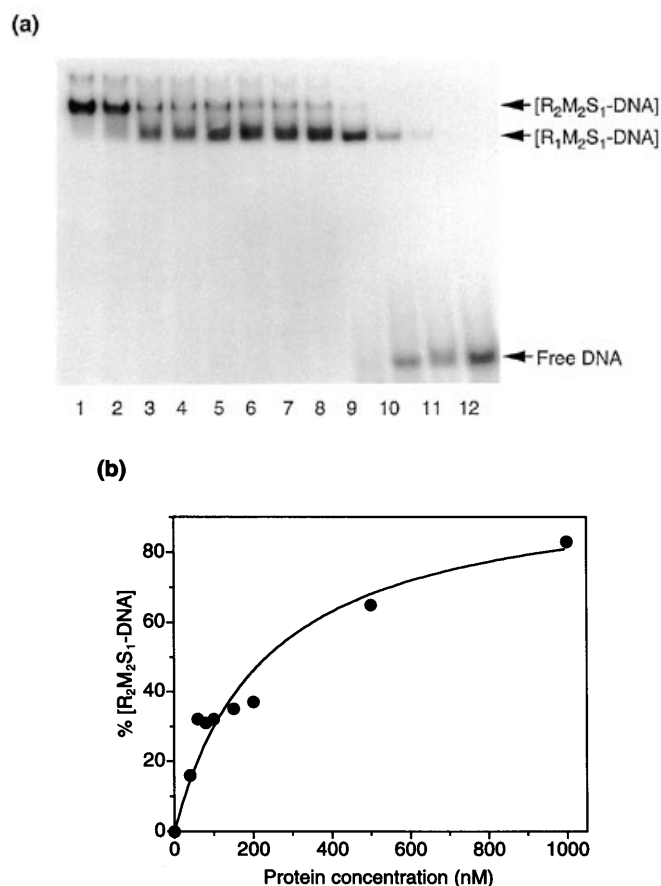


Figure 4. Dissociation of the *EcoR124I* endonuclease complex. Dissociation of $R_2M_2S_1$ into $R_1M_2S_1$ and HsdR was monitored by a gel retardation assay with the 39mer oligoduplex containing one *EcoR124I* recognition site. The endonuclease was reconstituted by mixing the MTase and HsdR in a molar ratio of 1:2. A series of dilutions of this mixture in a concentration range from 1000 to 5 nM was incubated with 20 nM DNA at room temperature for 10 min and subsequently analysed on a 5% non-denaturing polyacrylamide gel (a). Lanes 1–12 correspond to the following protein concentrations: 1000, 500, 200, 150, 100, 80, 60, 40, 20, 10, 5, 0 nM. The gel was quantified using a PhosphorImager and the percentage of $R_2M_2S_1$ -DNA complex of the total bound DNA was plotted against protein concentration (b).

HsdR subunit by itself shows only a weak DNA/Mg²⁺-dependent ATPase activity which is 100 times lower than that of the endonuclease (37). DNA cleavage appeared only when the HsdR:MTase ratio was >1 and the $R_2M_2S_1$ complex was formed (Fig. 6a). Both activities approached saturation level at an HsdR:MTase ratio of 4–5, where the gel retardation assay indicated that the $R_2M_2S_1$ species was fully present. These data indicate that only the $R_2M_2S_1$ species is capable of DNA cleavage.

DISCUSSION

The gel retardation and DNA cleavage experiments presented in this work revealed that the active form of the type IC *EcoR124I* restriction endonuclease has a subunit stoichiometry of $R_2M_2S_1$. It is assembled from the trimeric MTase (M_2S_1) and the HsdR subunits through a stable intermediate with a stoichiometry of $R_1M_2S_1$, which does not cleave DNA. While binding between the MTase and the first HsdR subunit is apparently very stable,

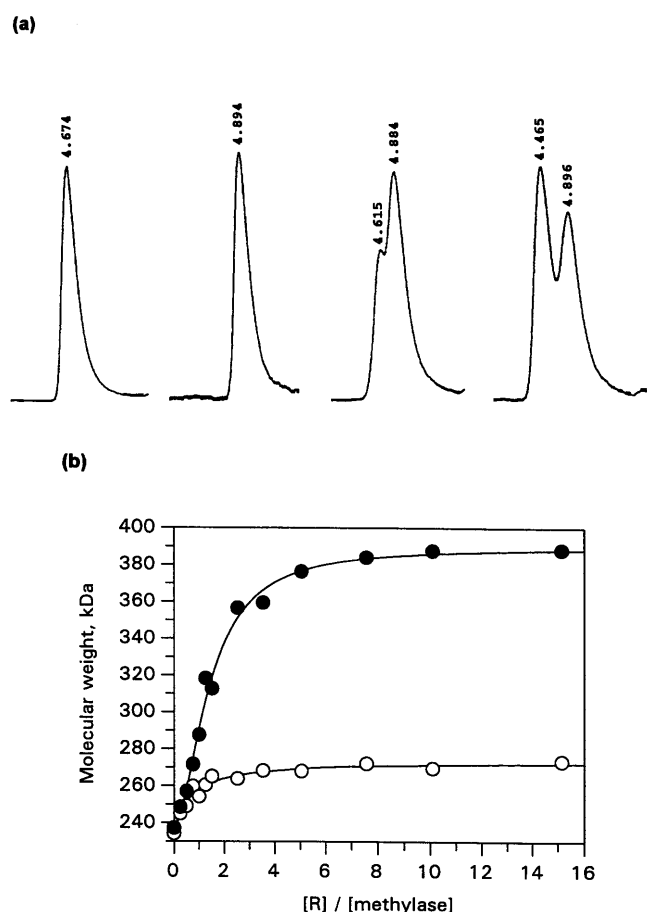


Figure 5. Elution profiles from the HPLC gel filtration column and titration of MTase with HsdR followed by HPLC gel filtration. (a) From left to right the traces show MTase (420 nM), HsdR subunit (1 μ M), MTase (420 nM) freshly mixed with a 5-fold excess of HsdR subunit, and MTase (420 nM) incubated with a 5-fold excess of HsdR subunit for 24 h before application to the column. The numbers are the elution times, in minutes, of the peaks. (b) The molecular weight, determined by HPLC gel filtration, of the complex formed between MTase and HsdR subunit as the HsdR subunit concentration is increased. The MTase concentration was 420 nM throughout. The samples were applied to the column immediately after mixing (○) or after 24 h incubation (●). The lines drawn are only to guide the eye.

interaction between the $R_1M_2S_1$ intermediate and the second HsdR subunit is much weaker. The $R_2M_2S_1$ complex dissociated into the $R_1M_2S_1$ complex and the HsdR subunit with an apparent K_d of $\sim 2.4 \times 10^{-7}$ M (Fig. 4).

In contrast to *EcoR124I*, the $R_2M_2S_1$ complex of *EcoKI* endonuclease, a member of the type IA family, is relatively stable and both HsdR subunits appear to be bound to the MTase with similar affinities (20; L.Powell and N.Murray, personal communication).

Our results indicate that the two HsdR subunits interact differently with the MTase in the assembly of the *EcoR124I* endonuclease. A simple model for assembly would be that there are two equivalent HsdR-binding sites on the MTase, but that the presence of one bound HsdR subunit affects the binding of the second HsdR subunit perhaps by partially blocking the second site, or by means of a conformational change at the second site. This model would require that the $R_1M_2S_1$ complex should be a 1:1 mixture of two species with occupation of either of the two

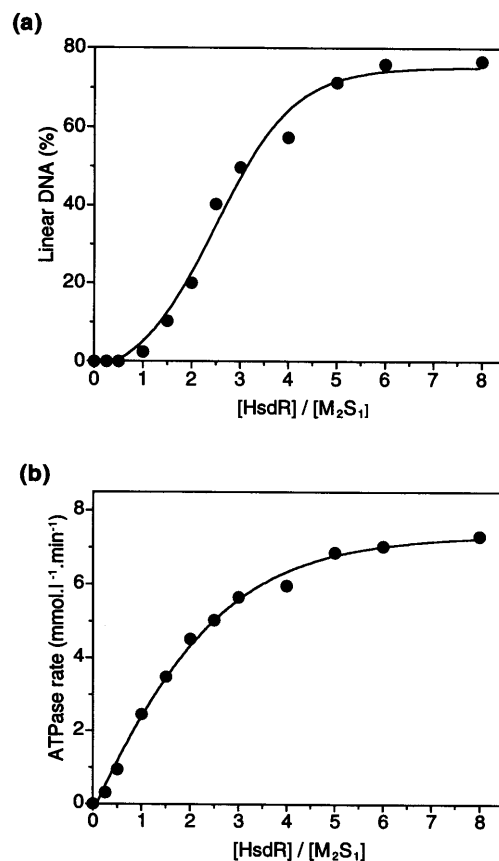


Figure 6. Titration of *EcoR124I* MTase with HsdR, followed by measurement of DNA cleavage and ATPase activity. A mixture of 200 nM MTase and 200 nM pDRM-1R DNA was incubated with increasing concentrations of HsdR in the presence of 0.2 mM AdoMet and 10 mM ATP. Concentration of linear DNA produced after 5 min incubation (a) and the rate of ATP hydrolysis (b) was plotted against HsdR:MTase molar ratio. Reaction products were quantified as described elsewhere (23). The lines drawn are only to guide the eye.

HsdR-binding sites. A second model would require that the two sites are not equivalent and that the assembly is inherently asymmetric with only one type of $R_1M_2S_1$ complex being formed. However, the data presented in this work are not sufficient to definitely determine which model is applicable.

Since binding of the first HsdR subunit to the MTase is much stronger than binding the second HsdR subunit to the $R_1M_2S_1$ intermediate, the *in vivo* formation of the $R_2M_2S_1$ complex will be dependent on the ratio between the HsdR subunit and the MTase in the cell. At an HsdR:MTase ratio of <1 , only the $R_1M_2S_1$ complex will be present. The $R_2M_2S_1$ complex will be formed only when the HsdR:MTase ratio is >1 . Indeed, when the ratio between MTase and HsdR was varied *in vivo* by using different recombinant two-plasmid systems, the resulting endonuclease preparations differed in ratio between the species (Fig. 1). The purified endonuclease produced from *hds* genes under the control of their natural promoters was also found to be a mixture of $R_1M_2S_1$ and $R_2M_2S_1$ species (J.Dreier, personal communication). This shows that the heterogeneity of the stoichiometry of *EcoR124I* endonuclease assembled *in vivo* was not a result of using an artificial expression system. The presence of two non-equivalent binding sites for HsdR has also been shown using the technique of surface plasmon resonance (38). This

clearly supports the data presented in this paper and has shown that the two sites differ in binding affinity by at least two orders of magnitude. Since two separate techniques have demonstrated the equilibrium between the $R_2M_2S_1$ and $R_1M_2S_1$ forms of the endonuclease, we believe the slow assembly of the R_2 complex in the absence of DNA, as measured by HPLC gel filtration, either reflects the experimental conditions used, or the absence of DNA greatly decreases the rate for attainment of thermodynamic equilibrium.

Why should the restriction endonuclease of the *EcoR124I* R–M system be such a weak complex and the stable assembly intermediate $R_1M_2S_1$ not be able to cleave DNA? We suggest this may reflect a very sensitive mechanism for regulation of restriction activity following conjugative transfer of the *hsd* genes into a non-modified recipient cell. The *EcoR124I* R–M system was originally found on the conjugative plasmid R124 (16) and this presents an unusual problem for the recipient cell. If the R124 plasmid entered a new host and immediately produced an active endonuclease, the unmodified host DNA would be restricted and the host cell would not survive. However, this does not happen and the host chromosome is modified following conjugation. Despite various attempts to show transcriptional or post-transcriptional control of this phenomenon for the *EcoR124I* R–M system (32; K.Firman, unpublished observations), there is no evidence of such control. Nor is there any evidence of such control for the other type I R–M systems. Therefore, we propose that this control is exercised at the level of subunit assembly. The M_2S_1 MTase is a very tight complex and it will be assembled first in the cell. Since the MTase can form a tight complex with one HsdR subunit, which does not cleave DNA, all the HsdR subunit will initially be trapped in this inactive complex. Therefore, the MTase (and possibly the $R_1M_2S_1$ complex) can modify and protect the host DNA. The weak endonuclease complex $R_2M_2S_1$ can be formed only when a large amount of HsdR has built up in the cell, which is unlikely during the early stage of establishing the R–M system in a new host. When cellular levels of individual subunits were altered by using different promoters and expression vectors, it was observed that excess HsdR over MTase was lethal, unless the MTase was already established in the cell (23). This implies that the function of the *EcoR124I* endonuclease in the cell is finely balanced and absolutely dependent upon subunit concentration. Control of restriction and modification via subunit assembly has also been recently proposed for the *EcoKI* restriction endonuclease (20), suggesting this mechanism may be common to all type I systems. In addition, the *EcoR124I* system shows a relatively high methylation activity with non-modified DNA (23) when compared to that of *EcoKI* (11). This will also ensure that a non-modified chromosome in the recipient cell can be readily modified following conjugal transfer of the *hsd* genes.

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